

Main points of the Ph.D. thesis

The role of autophagy in Myc-induced overgrowth

Péter Nagy

Biological Doctoral School, Leader of Doctoral School: Prof. Anna Erdei, DSc
Molecular Cell and Neurobiology Doctoral Program, Leader of Program: Prof. Miklós Sass, DSc
Eötvös Loránd University Faculty of Sciences



Supervisor: dr. Gábor Juhász, PhD, research associate professor

Eötvös Loránd University Faculty of Sciences
Department of Anatomy, Cell and Developmental Biology
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Introduction

Autophagy is an evolutionarily conserved, ancient lysosomal degradation pathway of eukaryotic cells. During autophagy, bulk material is transported to acidic autolysosomes for degradation through fusion of double membrane autophagosomes and lysosomes. Monomers produced by degradation are recycled for reuse in biosynthetic or energy-producing processes.

The selectivity of autophagic degradation is a widely disputed topic. p62/Ref2P and Alfy/blue cheese are selective cargo receptors of autophagy. p62 is a multidomain adaptor protein with an N-terminal PB1 (Phox and Bem1p) domain mediating its aggregation, an ubiquitin-binding UBA (ubiquitin-associated) domain, and an Atg8-interacting motif responsible for its selective degradation by autophagy. p62 binds to Keap1 (kelch-like ECH-associated protein 1) and disrupts the Keap1-Nrf2/cnc interaction, resulting in stabilization of Nrf2/cnc and induction of antioxidant responses.

Basal autophagy removes dysfunctional and superfluous organelles (e.g. mitochondria) from the cell. Long half-life and unfolded, toxic proteins are removed by autophagy as well, which plays an essential role in protein and organelle turnover this way, and maintains cellular homeodynamics.

Autophagy has a central role in many pathological and physiological processes such as neurodegenerative diseases, aging and cancer. Autophagy defects cause the accumulation of toxic protein aggregates in neurons, which leads to neurodegeneration and different proteinopathies (Alzheimer disease, Parkinson disease, Creutzfeld-Jacob syndrome). In the lack of autophagy, reactive oxygen species from damaged, dysfunctional, non-removed mitochondria induce oxidative stress. These reactive oxygen species are genotoxic and induce mutations which frequently lead to tumorprogression. Elevated Myc expression is associated whit different types of tumors: prostate, gastric, colon, breast cancer and melanoma. Myc is a transcription factor which binds to E-boxes in the core promoter region of genes, and activates transcription when complexed with Max.

Paradoxically, despite the tumor suppressor functions of autophagy, it may play a central role in the early steps of tumorigenesis. Before formation of proper vasculature in solid tumors, autophagy promotes tumor cell survival. In the later stages of tumorigenesis, autophagy may supply monomers for the increased glycolitic activity (Warburg effect) of tumor cells.

Autophagy has a key role in the homeodynamics and stress responses of the cells. Exploration of basic molecular mechanisms of autophagy is critical for developing novel therapeutic strategies for numerous diseases with dysregulated autophagy in the future.

There are many feasible approaches for the isolation of genes involved in autophagy: 1. we can use microarray chips for detection of fold changes in gene expression levels. Based on correlation of gene expression patterns, we may find new regulators of autophagy. The advantage of this approach is its rapidity but the results do not provide functional data. 2. RNAi-mediated gene silencing, and development of transgenic RNAi libraries allow us to carry out genome-wide screens in vivo for genes involved in autophagy. We can explore gene function based on loss of function phenotypes using a combination of RNAi-mediated gene silencing and other *Drosophila* genetic tools, such as mutants or overexpression of dominant-negative transgenes.

Methods

Genome-wide RNAi screen

We carried out a conserved genome-wide RNAi screen in mosaic larval fat bodies for genes involved in autophagy in *Drosophila*.

Microarray, QT-PCR

We measured gene expression changes using microarray chips and carried out QT-PCR experiments.

Recombinant DNA technology

We developed different plasmid constructs for identification of protein-protein interactions and generation of transgenic flies.

Cell cultures, immunoprecipitation (IP)

Protein-protein interactions were studied by IP carried out in *Drosophila* cell cultures.

Transgenic animals, genetics

We explored cellular processes and genes which are required for Myc-induced cell overgrowth using the *Drosophila* genetic system. We established the genetic relationship between genes/proteins by applying different combinations of fluorescent reporters, RNAi lines and overexpression lines.

Western blot

After co-immunoprecipitation we analyzed protein-protein interactions in Western blots. We measured autophagic activity in Western blots by detecting lipidated Atg8a and free mCherry.

Microscopy

We measured cell size, autophagic and antioxidant activity in fluorescent microscopy. We confirmed our findings regarding autophagic activity ultrastructurally as well.

Statistics

We evaluated our results using adequate statistical tests to determine the level of significance between quantitative traits.

Objectives

- Detect of changes in Atg gene expression levels in response to starvation using microarray chips.
- Carry out a genome-wide RNAi screen for genes involved in autophagy in *Drosophila*.
- Evaluate of Myc function during autophagy.
- Investigate the effect of Myc overexpression on autophagy, unfolded protein response and antioxidants in poliploid and diploid larval and adult tissues.
- Unfold the role of autophagy, unfolded protein response and antioxidant activity in Myc-induced cell overgrowth.

Results

- We showed in our microarray experiments that most of the autophagy-related genes are upregulated during starvation.
- Using our p62 antibody we showed that p62 accumulates in *atg* mutants.
- We tested 7400 RNAi lines in our genome-wide RNAi screen corresponding to 7100 conserved genes. We identified 213 genes required for autophagy. Well characterized Atg genes were positive hits in the screen as well.
- We detected defects in autophagosome lysosome fusion in Syntaxin 17 mutants, which led to neurodegeneration and locomotive defects.
- We demonstrated that the failure of proteasomal degradation led to hiperautophagy in cells.
- Myc oncogene was one of the most promising candidate hits in our screen, and was selected for further characterization.

- We found that the Myc transcriptional program is required for basal and starvation-induced autophagy, based on *Myc* null mutant, *Myc* RNAi and Mad overexpression studies (LysoTracker Red staining, anti-Atg8a and anti-p62 immunostaining, ultrastructural examination).
- Myc overexpression led to overgrowth of cells in poliploid and diploid larval and adult tissues (Figure1: A).

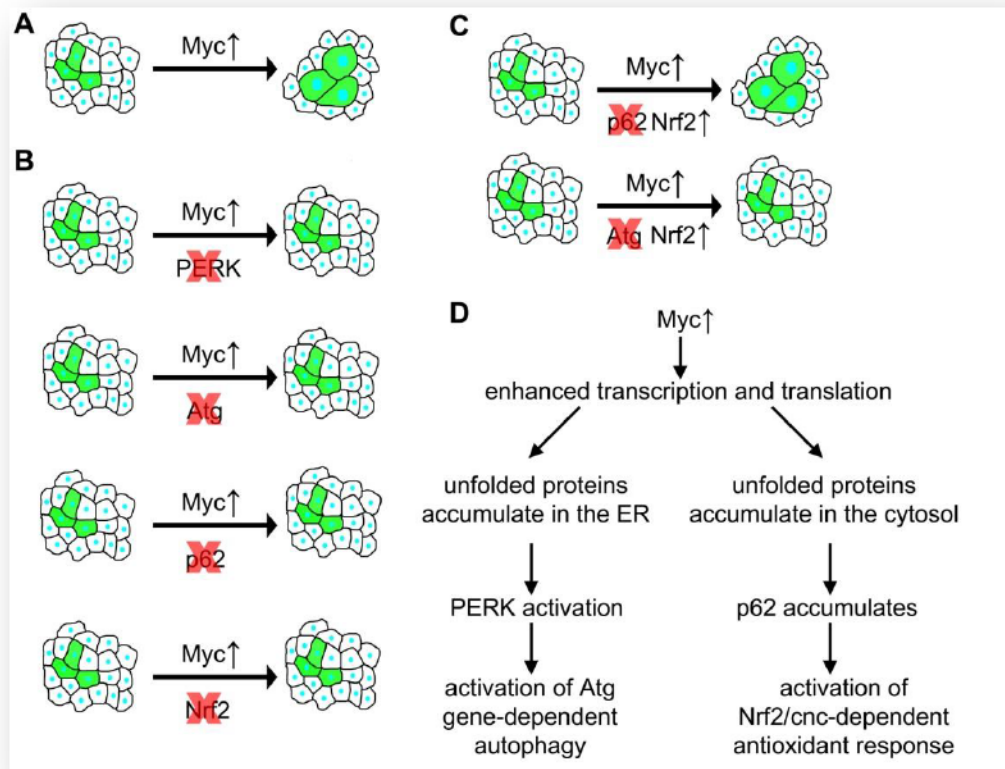


Figure 1. Summary of genetic interactions in the context of Myc-induced overgrowth

- Myc overexpression induced autophagy in poliploid and diploid larval cells (based on LysoTracker Red staining, mCherry-Atg8a, anti-Atg8a immunostaining, Western blot, ultrastructure).
- Forced Myc expression increased autophagic degradation rate, based on tandemly tagged Atg8a reporter and chloroquine treatment.
- Ubiquitinated proteins and p62 accumulated in cells during Myc overexpression. Elevated phospho-eIF2 α levels and Xbp1-GFP reporter expression indicated ER stress.
- PERK overexpression was sufficient to induce autophagy in well-fed larvae.

- Myc overexpression elevated antioxidant activity (GstD-GFP and GstD-lacZ reporters).
- Drosophila p62 bound to Keap1 similar to mammals.
- Block of PERK signaling by *PERK* RNAi or Gadd34 overexpression suppressed Myc-induced cell overgrowth (Figure 1: B).
- Myc-induced overgrowth was suppressed by chloroquine treatment, which is a non specific autophagy inhibitor.
- Genetic block of autophagy completely suppressed Myc-induced overgrowth (Figure 1: B).
- Genetic inhibition of p62/Nrf2/cnc signaling suppressed Myc-induced overgrowth. (Figure 1: B).
- Nrf2/cnc overexpression successfully rescued *p62* loss of function phenotypes, while it had no effect on loss of autophagy during Myc overexpression. We thus concluded that Myc overexpression induces autophagy and antioxidant responses in parallel to each other (Figure 1: C).

Main points of the thesis

1. *Atg* genes are transcriptionally upregulated in response to starvation.
2. The Myc transcriptional program is required for basal and starvation-induced autophagy.
3. Myc overexpression not only leads to overgrowth of cells, but is also elevates unfolded protein response (UPR) including ER stress due to strongly enhanced transcription and translation in the cells (Figure 1: D).
4. Mechanistically, activated ER stress induces autophagy through PERK signaling during Myc overexpression (Figure 1: D).
5. UPR triggered by forced Myc expression increases p62 levels which activates Nrf2/cnc dependent antioxidant responses (Figure 1: D).
6. Forced Myc expression activates autophagy and antioxidant response in parallel to each other (Figure 1: D).
7. Autophagy, UPR, p62 and antioxidant activity are all required for Myc-induced cell overgrowth (Figure 1: B).

Outlook

- Cancer cells depend on oncogenes that originally triggered tumor initiation and progression. This phenomenon is known as oncogene addiction. These types of addiction pathways are logical and obvious targets of numerous cancer therapies. Inhibition of oncogenic Myc is difficult as Myc is a transcription factor without an easily druggable active center, and its function is also indispensable for normal cells. Novel targets of cancer therapies could be those cellular processes which are required for the maintenance of tumorigenic state (e.g. tumor cell survival), but dispensable in normal cells. These are the largely unexplored non-oncogene addiction pathways. Myc-induced autophagy, UPR and antioxidant responses may represent genuine non-oncogene addiction pathways, which could be promising therapeutic targets.
- The lysosomotropic agent chloroquine was commonly used in malaria treatment and it could be a potential drug during Myc-associated tumor therapies (Amaravadi, Yu et al. 2007; Maclean, Dorsey et al. 2008). Autophagy induced by transient Myc expression was successfully suppressed in mouse embryonic fibroblast cells by 4-PBA (increases chaperone activity) and bafilomycin A1 (blocks fusion of autophagosomes and lysosomes) (Hart, Cunningham et al. 2012). We successfully suppressed the cellular overgrowth induced by overexpression of different copies of *Drosophila* Myc by genetic manipulation or chloroquine treatment as well. This strongly supports further clinical trials of autophagy inhibitors in the case of Myc-associated tumors.
- Based on our data, elevated Myc levels may even turn out to be useful as a biomarker before therapeutic application of inhibitors for key autophagy, UPR or antioxidant proteins in cancer patients. This may provide a tremendous benefit in personalized therapies.

Publications

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*shared first authorship

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